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(54) Title: PRE-IMPLANTATION SCREENING

(57) Abstract

The embryo of a putative transgenic animal can be screened for the presence of an integrated transgene, by removing non-integrated DNA from one or more cells of the embryo, for example by a fixing/washing procedure in which the fixing agent is preferably formaldehyde, and analysing the embryonic genome in the said cell or cells for the presence of the transgene. The analysis is preferably conducted using the polymerase chain reaction, using primers specific for the transgene.

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PRE-IMPLANTATION SCREENING

This invention relates to improvements in the production of transgenic animals.

Genetic alterations to mammalian species can be achieved following the microinjection of genes into the pronuclei of single-cell embryos (Brinster *et al*, *Cell* **27** 223-231 (1981)). Successful gene transfer, dependent on the incorporation of exogenous DNA into the host chromosome, only occurs in a proportion of the injected embryos. These resultant offspring, termed transgenic, will normally carry the transgene in a varying proportion of somatic and germ tissues and transfer them to their own offspring. Transgenic biology has provided new opportunities to investigate numerous aspects of gene structure and expression, and, more recently, the application of this technology to farm animals has been used to modify quantitative traits, although this has proved problematical (sheep: Rexroad *et al*, *J. Reprod. Fertil.* **41** 135-146 (1990, Supplement); Murray *et al*, *Reprod. Fertil. Dev.* 1 242-248 (1989); pig: Pursel *et al*, *J. Reprod. Fertil. Suppl.* **41** 77-87 (1990)).

Extensive studies with transgenic mice have identified the 5' and 3' regulatory sequences of the gene encoding the major whey protein of ovine milk, β-lactoglobulin (BLG). Transgenic sheep have been produced in which the expression of BLG promoter/fusion genes is directed in a tissue specific manner to the mammary gland. These studies have created new opportunities in biotechnology as human proteins of pharmaceutical and commercial interest can be synthesised in the mammary gland and later isolated from milk.

Since sheep milk contains about 40g/l of endogenous protein, there is clearly potential for the high level expression of foreign secretory proteins in milk. The introduction of fusion genes that code for the proteins, human factor IX and α_1-antitrypsin (AAT), driven by the promoter of the major ovine whey protein, BLG, has been found to be an effective method for the synthesis, processing, and recovery of these proteins in milk (Clark *et al*, *Bio/Technology* 7 487-492 (1989)). Indeed recently, transgenic sheep carrying the human AAT gene have been produced where AAT is the major milk protein (Wright *et al*, *Bio/Technology*, 9 830-834 (1991)). This use of the mammary gland, as a "bioreactor" for the production of pharmaceutical proteins, is of commercial interest and is a new and developing area in biotechnology. However, it should also be noted that the same technology could be used for producing modified milks for human consumption.

Unfortunately, the production of large domestic transgenic animals is very expensive in both time and money as large numbers of animals are required to produce donor embryos and to act as recipient animals for the injected embryos. The expense is compounded by the fact that for large animals, there is a very low incidence of transgenic births when compared to the numbers of embryos injected. At the present time, whilst 15-25% of the offspring of transferred mouse embryos are transgenic, the figure is considerably lower for sheep and pigs (0.5-5%). This lower incidence of transgenesis that has been consistently observed with domestic species (Simons *et al*, *Bio/Technology* 6 179-183 (1988); Murray *et al*, *Reprod. Fertil. Dev.* 1 242-248 (1989); Krimpenfort *et al*, *Bio/Technology* 9 844-847 (1991)) is acknowledged to be

due to methodological problems associated with the isolation of embryos, difficulties associated with microinjection (eg opacity of embryos), and the quality of *in vitro* embryo culture techniques. A further problem is that the injected genes are usually integrated as multiple copies at a single locus and the site of insertion into the host's chromosomal DNA is believed to be random. A consequence of this random insertion is that, with very few exceptions (eg the "dominant control" regions of globin (Grosveld *et al*, *Cell* 51 975-985 (1987)), CD2 (Greaves *et al*, *Cell* 56 979-986 (1989)), and lysozyme (Bonifer *et al*, *EMBO J.* 9 2843-2848 (1990)) expression from integrated sequences is dictated by the local chromosomal environment and tends to be unpredictable and highly variable in different individuals. This has the unsatisfactory consequence that larger numbers of transgenics have to be generated in order to have the probability of obtaining one high yielding, founder animal.

Clearly, for logistical, economic, and commercial reasons, it is desirable to increase the efficiency of large transgenic animal production. In the short term, the most direct way of achieving this will probably be to use pre-implantation screening methods to identify embryos that carry integrated transgenes, prior to their transfer to recipient animals. Screening would reduce costs of purchase and maintenance of large numbers of animals.

A useful screening method will be one that, ideally, led to transgenic offspring only; second best, but still useful, would be a screening method which reduced the number of non-transgenic animals being born. To do this,

the screening method must be able to differentiate between integrated and non-integrated DNA. To enable the method to distinguish between integrated and non-integrated DNA, either the two forms of DNA must have a distinct physical difference or they must be separated in some way.

Several attempts have been made to establish a pre-implantation screening technique. However, one major hurdle has always been the short time available for screening. After biopsy there is generally only 1-2 days during which screening can be performed before the biopsied embryos have to be transferred to a recipient animal. Therefore, the screening method must combine accuracy with speed.

Detection of transgenesis has been attempted by performing *in situ* hybridisation (ISH) on cells biopsied from bovine embryos (WO-A-9222647). However, the small window between biopsy and embryo transfer means that the biopsied cells can not be synchronised and induced into metaphase in time, and so the ISH is performed on interphase nuclei. This means that the chromosomes cannot be seen adequately and it is very difficult to be certain that integration has occurred. ISH also involves a great deal of technical expertise and equipment and it would be difficult to accommodate a lot of samples on a routine basis into the screening schedule.

The polymerase chain reaction (PCR; Saiki *et al*, *Science* **239** 487-491 (1988)) promotes the synthesis and exponential amplification of specific regions of DNA bordered by pre-selected oligodeoxynucleotide primer sequences. One of its many uses is the detection and

screening for the presence of genes in a small number of cells taken from the early embryo of established transgenic lines (King and Wall, *Mol. Rep. Dev.* 1 57-62 (1988)). However, the application of PCR to identify the fate of DNA sequences injected into single-cell embryos (Ninomiya *et al*, *Mol. Rep. Dev.* 1 242-248 (1989)) has been compromised by the observation that in most injected murine embryos, sequences which have been introduced by injection persist in a non-integrated state for at least 4-5 days of development (Burdon and Wall, *Mol. Rep. Dev.* 33 436-442 (1992)). To be successful, a PCR technique would have to be able to distinguish integrated from non-integrated sequences. At present no known PCR technique can do this on a routine and regular basis. However, the speed at which results can be generated using PCR make it an extremely attractive method.

One PCR technique which has been advocated (WO-A-9108216 and WO-A-9222647) is based on the assumption that integration and replication of a transgene methylated in a prokaryotic fashion causes the transgene to become methylated in a eukaryotic fashion, which can be tested for by use of methylation-sensitive enzymes. This method, and others which rely on one or more enzyme-mediated steps, such as inverse PCR, are dependent on the enzyme reacting with 100% efficiency. If some target molecules in the reaction are not altered by the enzyme (digested, ligated, etc) then the PCR step, which will have been optimised for high stringency, will produce a lot of false negatives or positives.

The present invention seeks to provide a solution to the problem of pre-implantation screening in the production of transgenic animals. It has been found that an

amplification process, such as the PCR reaction, can form the basis of a useful pre-implantation embryo screening method if the non-integrated DNA in the embryo is removed, for example by suitable washing procedures. This seemingly simple procedure forms an attractive alternative to the complexities of the process of WO-A-9222647.

According to the present invention, there is provided a method of screening the embryo of a putative transgenic animal for the presence of an integrated transgene, the method comprising removing non-integrated DNA from one or more cells of the embryo and analysing the embryonic genome in the said cell or cells for the presence of the transgene.

Typically, in the practice of the invention, a DNA construct containing the transgene (the "transgene construct") sought to be introduced into an animal will be microinjected or otherwise introduced into a single embryonic cell such as a single cell egg or embryonic stem cell. Other means of introduction include but are not limited to electroporation, viral transfection, transformation (for example mediated by calcium phosphate or liposomes), ballistics, homologous recombination and nuclear transfer.

The procedure will generally contain a number of replicates. One or more copies of the transgene construct, it is to be hoped, will integrate into the genome of at least some of the cells; others will remain in the cell, unintegrated.

The fertilised egg will then usually be allowed to grow, *in vitro* or *in vivo*, to an appropriate stage. For mice or other small animals, this growth may be allowed to take place *in vivo*. For cattle, sheep and other large animals, it may well be preferred for the growth to take place *in vitro*. It may be that up to half the cells may be taken from the four cell stage, but the most appropriate stage to which the embryo is allowed to develop will usually be from the 16 cell embryo to early blastocyst stage; the morula stage (16 to 32 cells) is preferred. The number of cells present in the embryo at the various stages will vary from species to species.

The number of cells to be taken from the embryo will generally be in the order of one quarter to one third of the cells present in the embryo. This proportion usually ensures that there are sufficient cells left in the embryo for continued viability yet provides enough for meaningful analysis. Typically, from 2 to 8 cells will be taken.

From the abstracted cells, non-integrated DNA is removed. This may be achieved by fixing and washing, generally with a suitable fixative and water (which will preferably be pure, for a reason to be discussed below). Suitable fixatives which can be used, alone or in combination, include, but are not limited to, formaldehyde, paraformaldehyde, glutaraldehyde, methanol, acetic acid, ethanol, acetone and air drying. Fixation can be performed with or without a protein digestion step using, for example, pronase, proteinase K or other protease(s).

Fixation chemistry has been shown to affect the detection of PCR-amplified DNA in peripheral blood monocytes.

Nuovo *et al*, *PCR Methods and Applications* 2(4) 305-312 (1993)) were trying to enhance the *in situ* detection of PCR-amplified DNA and looked for conditions to cross-link and fix it. However, they also found conditions which completely removed the PCR-amplified DNA. The applicability of this technique to the problem of pre-screening putative transgenic embryos was certainly not foreshadowed.

After the non-integrated DNA is removed, the genome is analysed for the presence or absence of the transgene. While in principle a variety of methods can be used in this step of the method of the invention, in practice it is preferred to use a DNA amplification procedure, which will generally be sequence-specific. The polymerase chain reaction (PCR) meets this criterion and is preferred.

PCR amplification of DNA may be initiated with primers chosen from, or complementary to, the transgene construct. Typically, a pair of primers will be chosen for DNA chain synthesis on complementary strands in opposing and converging directions. The resulting double strand amplification product may then be separated on the basis of size from the other DNA in the sample (for example using agarose gel or other electrophoresis): from knowing the relative positions of the primers, the predicted size of the PCR amplification product can be established, and it is merely necessary to look for DNA of an appropriate size on the gel or other sizing means. If further confirmation of the sequence of the amplified product is required, it may be possible to digest the PCR product using an appropriate restriction enzyme. If the DNA sequence is known, fragments of known size will be

generated. Alternatively, the PCR product may be hybridised (for example by Southern blotting) with the original PCR primers.

Primer sequences will be chosen from the transgene construct sequence with the following guidelines in mind:

- the amplified product should be appropriately sized for convenient sizing (for example by agarose gel electrophoresis;
- unique sequences should be chosen; and primers with 3' overlap in particular should be avoided;
- sequences with significant secondary structure, such as palindromic sequences, which can lead to hairpin loop formation should be avoided; and
- the AT:GC ratio, which determines the melting temperature (T_m) of the DNA duplex, should be similar to that of the fragment being amplified and the T_m of the two primers should be alike (Erlich, H. A. Ed., "PCR Technology", Stockton Press, 1989).

If the PCR primers are sensitive enough, the DNA may, after the optional protease digestion step, be divided into two or three aliquots, in separate tubes or other suitable containers. This allows duplicate PCRs to be performed, for extra accuracy. Alternatively, one or more control primers which are specific to an endogenous gene, such as hypoxanthine phosphoribosyl transferase

(HPRT) or bovine trophoblast protein (bTP) in the case of cattle, may be used. Such a primer would be a control for the PCR, ensuring that sufficient DNA were present and that all the reagents were functional. The use of construct DNA in a positive control should preferably be avoided because of the increased possibility of false positives. (It is not recommended that both or all sets of primers, if there are more than one, be used in the same reaction; one set invariably out-performs the other, leading to ambiguous results.) Another option is to use one of the duplicate DNA samples to sex the embryo, using suitable PCR primers.

Having regard to the above considerations, it will be apparent that the exact nature of the PCR primers will depend primarily on the sequences available in the transgene construct and secondarily on the practical factors which affect the course of PCR reactions.

While the PCR or other transgene analysis is taking place on a small number of cells of the embryo, the remainder of the embryo is kept viable by appropriate incubation, either *in vivo* (which may be preferred for small animals such as mice) or *in vitro* (which may be preferred for large animals such as sheep, pigs and cattle). If the transgene analysis yields a negative result, the embryo may simply be discarded. However, if the transgene analysis shows that the embryo's genome has the desired transgenic and optionally other (for example, sex) characteristics, the embryo may be implanted or re-implanted into a (foster) mother; the methodology for this is well established and routine. Thereafter, the embryo is allowed to develop to term and birth takes place in the normal way.

The invention is not particularly limited to any particular species of animal, although it will have primary applicability for warm blooded, placental mammals including mice, cows, sheep, goats, rabbits and pigs.

The invention will now be illustrated by the following examples. The examples refer to the accompanying drawing, in which:

FIGURE 1 shows a partial map of the structure of the construct AATB and gives the location of the PCR primers 736R and 786V.

EXAMPLE 1 - Demonstration that Integrated Sequences are PCR-Amplifiable after Fixation

Mouse eggs from mouse transgenic line 46-2 (Carver *et al*, *Bio/Technology*, 11(11) 1263-1270 (1993)) were treated as follows; this line was estimated to give 2-4 copies of the integrated AATB transgene per cell. (AATB is a "minigene" construct encoding human α_1-antitrypsin and including some but not all of the introns present in the normal, full length human α_1-antitrypsin gene.) The eggs were cultured at 37°C, 5% CO_2, 95% air with ~100% humidity. The cultured eggs were removed at the compacted morula (CM) stage and transferred to a siliconised glass depression slide containing 4% formal saline (BDH) for 30 mins. (4% formal saline is a histological fixative, available under the trade mark GURR from British Drug Houses, comprising 4% w/v formaldehyde and 0.9% w/v sodium chloride in water.) During this time the zonas on the eggs disappeared, presumably having been dissolved. The formal saline was removed and replaced with water; this step was repeated 3 times.

The fixed eggs were individually transferred to a dish containing high quality water (resistivity at 25°C >15MΩ.cm) that had been irradiated with ultra-violet (UV) light for 3 minutes. An egg was removed into 10μl volume of UV-irradiated water and transferred into 500μl sterile centrifuge tubes and stored frozen at -20°C. Control injected or non-injected eggs were individually removed to UV-irradiated water as above, before storage at -20°C.

Tubes containing the eggs were removed from the freezer and allowed to thaw in a Class II environment cabinet fitted with a UV light. Liquid paraffin was added to each tube and the samples incubated at 94°C for 10 minutes followed by cooling to 80°C. The heating block was held at this temperature whilst the tubes were taken to the Class II cabinet. A PCR reaction mix complete with the PCR primers 736R and 786V and *Taq* polymerase (BM), using 1.25 units of polymerase and standard concentrations of other reagents (Erlich, H. A. Ed., "PCR Technology", Stockton Press, 1989), was added to each tube. Primers 736R and 786V have the following sequences:

736R: 5'-CAC TCC CTG CAG AGC TCA GAA GC-3'

786V: 5'-CTG TTG GAC TGG TGT GCC AGC TG-3'

and the following locations in relation to the AATB sequence:

736R: 801-823

786V: C' to 1029-1051

as shown in Figure 1; the predicted size of the PCR product is 250 bp.

The tubes were returned to the heating block and left for 1 minute before commencing the following programme:

20 x 94°C	20 secs	
60°C	15 secs	
71°C	10 secs	followed by
20 x 94°C	15 secs	
60°C	10 secs	
71°C	10 secs	1 sec. incremental for each temp. on each cycle
1 x 72°C	10 min.	

On completion of the programme (usually ~2.5 hours) the tubes were removed and a sample of each subjected to agarose electrophoresis. The stained gels were photographed.

Initial minor problems encountered with the assay were attributable to contaminating amplifiable DNA sequences. A combination of the introduction of filter-plugged, aerosol resistant tips, recirculating Class II microbiological environment cabinets and great caution and attention to detail when setting up, so as to avoid contamination, has eliminated this problem.

The results from mouse transgenic line 46-2 are presented in Table 1.

TABLE 1

Observed Transgenic Frequency Line 46-2

Control Eggs	%Transgenic Frequency	Fixed eggs	%Transgenic Frequency
36/57	64%	20/56	36%

These data demonstrate that the integrated sequences are PCR-amplifiable after fixation. The eggs were sourced

and processed at different times, which may be reflected in unequal distribution of the data. It may also be an indication of the reproducibility of such an assay accurately to reflect the distribution of an integrated transgene.

EXAMPLE 2 - Demonstration that Mouse Eggs Microinjected with AATB are Similar to those of the 46-2 Cell Line of Example 1

Normal mouse eggs were microinjected with AATB (Archibald *et al. Proc. Nat'l. Acad. Sci. USA* 87 5178-5182 (1990) at 6μg/ml. The injected eggs were processed as for the AATB 46-2 cell line eggs, as described in Example 1. The total number of eggs screened was 152 (fixed eggs + non-fixed eggs). The distribution of the transgene is presented in Table 2.

TABLE 2

Observed Transgenic Frequency - Injected Eggs

Injected Eggs	%Transgenic Frequency	Injected Eggs Fixed	%Transgenic Frequency
60/72	83%	23/80	29%

These data support the idea that the fixation procedure does reduce the number of PCR-amplifiable positive eggs. The intensity of the band produced from an injected non-fixed egg is very high and consistent between eggs, ie they all contain significant quantities of DNA. After fixation the intensity of any bands seen tends to be lower and sometimes quite faint. There is little if any consistency of intensity between eggs; ie the eggs are different. The transgenic frequency after fixation is

slightly higher than the observed live birth figure for this construct (~25%) and may reflect losses *in vivo* upon transfer of injected eggs to recipient females. Alternatively, the observed difference of 25%-29% may be the result of the number of eggs analysed *in vitro*.

In conclusion, the fixation/PCR procedure as outlined, reduces the amount of non-integrated DNA that is available for PCR-amplification. The observed reduction is not the same for each egg, and the overall reduction is approaching the observed transgenic frequency (live births) for this construct.

CLAIMS

1. A method of screening the embryo of a putative transgenic animal for the presence of an integrated transgene, the method comprising removing non-integrated DNA from one or more cells of the embryo and analysing the embryonic genome in the said cell or cells for the presence of the transgene.

2. A method as claimed in claim 1, wherein the embryo of the putative transgenic animal was prepared by microinjecting a DNA construct containing the desired transgene (the "transgene construct") into a single embryonic cell.

3. A method as claimed in claim 1 or 2, wherein one or more cells are removed from the embryo for DNA analysis when the embryo is at a stage of development from four cells to early blastocyst.

4. A method as claimed in claim 3, wherein the said cells are removed when the embryo is at the morula stage of development.

5. A method as claimed in any one of claims 1 to 4, wherein from one quarter to one third of the cells present in the embryo are removed for DNA analysis.

6. A method as claimed in any one of claims 1 to 5, wherein non-integrated DNA is removed by fixing and washing.

7. A method as claimed in claim 6, wherein formaldehyde, paraformaldehyde, glutaraldehyde, methanol,

acetic acid, ethanol, acetone and/or air drying is used as a fixing means.

8. A method as claimed in claim 6 or 7, which includes a protein digestion step.

9. A method as claimed in claim 8, wherein the protein digestion step involves the use of pronase or proteinase K.

10. A method as claimed in any one of claims 1 to 9, wherein the genome analysis is undertaken by a sequence-specific DNA amplification procedure.

11. A method as claimed in claim 10, wherein the amplification procedure involves a polymerase chain reaction (PCR).

12. A method as claimed in any one of claims 1 to 11, wherein the animal is a mouse, cow/bull, sheep, goat, rabbit or pig.

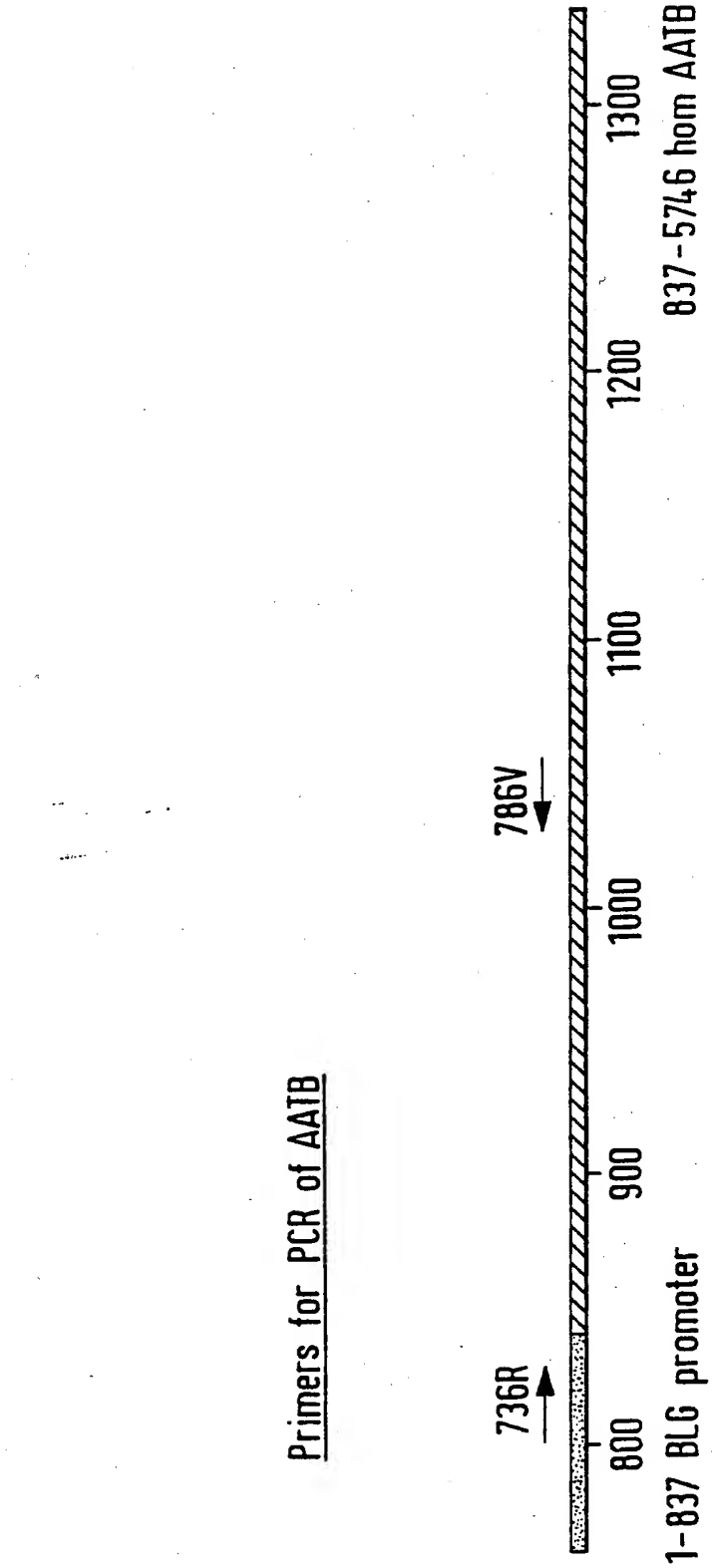
Primers for PCR of AATB
736R
786V
800
900
1000
1100
1200
1300
1-837 BLG promoter
837-5746 hom AATB
—736R 5'- CAC TCC CTG CAG AGC TCA GAA GC- 3' (801-823)
—786V 5'- CTG TTG GAC TGG TGT GCC AGC TG- 3' (C, to 1029-1051)

FIG. 1

INTERNATIONAL SEARCH REPORT

Internat · l Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01K67/027 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 22647 (GENPHARM INT.) 13 December 1992 cited in the application See whole document ---	1-12
A	MOL. REPROD. DEV., vol.33, no.4, December 1992, USA pages 436 - 442 T.G. BURDON ET AL. 'Fate of microinjected genes in preimplantation mouse embryos' cited in the application See the whole document ---	1-12
A	WO,A,93 22432 (GENZYME CORP.) 11 November 1993 See example 1 and claims ---	1-12
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
23 February 1995	04. 04. 95

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INTERNATIONAL SEARCH REPORT

Interna[illegible]l Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB,A,2 224 836 (ANIMAL BIOTECHNOLOGY CAMBRIDGE LTD) 16 May 1990 ---	1-12
A	PCR METHODS APPL., vol.2, no.4, May 1993, USA pages 305 - 312 NUOVO GJ ET AL. 'Importance of different variables for enhancing in situ detection of PCR-amplified DNA' cited in the application See the whole article -----	

1

INTERNATIONAL SEARCH REPORT

mation on patent family members

Interna' ıl Application No
PCT/GB 94/02413

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9222647	23-12-92	NONE	
WO-A-9322432	11-11-93	AU-B- 4225593	29-11-93
GB-A-2224836	16-05-90	NONE	